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Protein kinases A and C phosphorylate purified Ca^{2+}-ATPase from rat cortex, cerebellum and hippocampus[1]

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Abstract

The plasma membrane Ca^{2+}-ATPase (PMCA), the enzyme responsible for the maintenance of intracellular calcium homeostasis, is regulated by several independent mechanisms. In this paper we report that the protein kinases A and C differentially activate the Ca^{2+}-ATPase purified from synaptosomal membranes of rat cortex, cerebellum and hippocampus. The effect of protein kinases was more pronounced for the cortical enzyme, whereas cerebellar and hippocampal Ca^{2+}-ATPases were activated to a lesser degree. The preparation of Ca^{2+}-ATPase contained the phosphoamino acids, i.e., P-Ser and P-Thr, indicating that the enzyme was purified in phosphorylated state. The phosphorylation of Ca^{2+}-ATPase by PKA and PKC increased the amount of phosphoamino acids, but in a region-dependent manner. Using the specific antibodies against N-terminal portion of four main PMCA isoforms we have characterized the isoforms composition of Ca^{2+}-ATPase purified from the nervous endings of examined brain areas. Our results indicate that the activity of calcium pump is related to its phosphorylated state, and that the phosphorylation is region-dependent. Moreover, the differences observed could be related to the composition of PMCA isoforms in the different brain areas. Phosphorylation of the plasma membrane Ca^{2+}-ATPase appears to be a mechanism to control its activity. The results support also the possible involvement of PKA and PKC.

Keywords: Ca^{2+}-ATPase; Rat brain; Protein kinase A; Protein kinase C; Phosphorylation

1. Introduction

The plasma membrane calcium pump (PMCA) is responsible for ATP-powered returning of $[Ca^{2+}]_i$ to a basal level after depolarization of the neuronal cell. This is critical for the maintenance of membrane conductance, neuronal excitability and intracellular signaling. The enzyme is encoded by four genes that can be alternatively spliced to produce more than 30 transcripts [1,2]. A number of Ca^{2+}-ATPase mRNAs is generated in the brain and some of them show a region-specific expression, thus various types of neurons may use different combinations of PMCA isoforms [3,4]. The distribution of Ca^{2+}-ATPase protein in rat brain analyzed using immunological methods confirmed the regional and cellular diversity in localization of the main isoforms, as well as spliced variants of the enzyme [5,6]. It has been also shown that only neurons were PMCA-positive.

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Electron microscopic cytochemical study revealed that in rat brain the Ca^{2+}-ATPase was associated with synaptic membranes [7]. Synaptic terminals are the main point of communication between neurons, and possess all systems responsible for transduction of neurochemical and electrical signals. Therefore, the isolated synaptosomal membranes are a suitable and frequently used model for the study on neurotransmission, including Ca^{2+} signaling [8].

The activity of the Ca^{2+}-ATPase is regulated by several mechanisms, i.e., stimulation by calmodulin, acidic phospholipids, proteolytic cleavage or oligomerization (for review see [1,9]). In cells the plasma membrane calcium pump has been found to be activated by protein kinase A and protein kinase C [10–14]. In erythrocytes, PKA increased V_{max} and K_m for Ca^{2+}, whereas PKC increased V_{max} without changing the affinity of the pump for Ca^{2+} [10,15]. Up to the present, only partial data have been published on the regulation of calcium pump by phosphorylation processes in a nervous tissue. We have previously shown that Ca^{2+}-ATPase activity in rat cortical and cerebellar synaptosomal membranes was affected in vitro by treatment with PMA or cAMP [16]. Moreover, the enzyme purified from cerebellar membranes was a substrate for PKC-catalyzed phosphorylation [17]. The aim of the presented studies was to compare the effects of phosphorylation processes mediated by protein kinases A and C on the activity of Ca^{2+}-ATPase isolated from synaptosomal membranes of structurally and functionally different regions of adult rat brain, i.e. cortex, cerebellum and hippocampus.

2. Materials and methods

2.1. Reagents

All reagents used were of analytical grade. Reactive Red 120-Agarose, CaM-Agarose, egg yolk phosphatidylcholine, monoclonal antibody clone 5F10, antibodies against P-Thr and P-Ser, goat anti-mouse and mouse anti-rabbit IgG-coupled alkaline phosphatase conjugates, PMA (phorbol 12-myristate,13-acetate), and ATP were purchased from Sigma (Germany). Protein Assay kit was from BioRad (USA). The catalytic subunit of protein kinase A, rat brain protein kinase C, Immuno-blot assay kit, protease inhibitors (leupeptin, pepstatin, aprotinin) were from Calbiochem (Switzerland). $C_{12}E_8$ (octaethylene glycol mono-*n*-dodecyl ether) was from Nikko (Japan). [γ-^{32}P]ATP (10–30 Ci/mmol) was from Amersham (UK). Immobilon PVDF membrane was purchased from Millipore (Austria).

2.2. Purification of Ca^{2+}-ATPase

2.2.1. Preparation of synaptosomal membranes

Synaptosomal membranes of cerebral cortex, cerebellum and hippocampus from 3-month-old Wistar rats were prepared by the method of Booth and Clark, using Ficoll gradient centrifugation [18]. All used buffers contained 1 mM PMSF, 1 μM pepstatin, 10 μM leupeptin, 0.5 mM EGTA and 0.5 mM EDTA. The purified synaptosomal membranes were resuspended in 10 mM Tris–HCl, pH 7.4 and stored at −80°C until use.

2.2.2. Column chromatography

The procedures were as described previously [19]. In brief, the synaptosomal membranes were rapidly thawed and solubilized with 0.5% C_{12} E_8 in buffer A containing: 50 mM Tris–HCl (pH 7.4), 0.5 mM $MgCl_2$, 2 mM DTT, 20% glycerol, 0.5 mM EGTA, and 50 μM $CaCl_2$. Protein concentration was 4.0–4.5 mg/ml. Solubilization was performed for 10 min in an ice bath, and then the samples were centrifuged at 100 000 × *g* for 60 min (rotor SW 28, Beckman). The supernatant was supplemented with aprotinin (100 kallikrein units/ml) and loaded onto Reactive Red 120-Agarose column equilibrated with buffer B containing: 10 mM Tris–HCl (pH 7.4), 0.5 mM $MgCl_2$, 2 mM DTT, 20% glycerol, and 0.5% $C_{12}E_8$. Proteins were eluted with a step gradient of NaCl (0.5 M and 2 M) in buffer B, with the flow rates 0.2 ml/min. Fractions which contained the enzyme were eluted with 0.5 M NaCl. They were pooled, adjusted to contain 100 mM KCl, 50 μM $CaCl_2$ and 400 μg/ml phosphatidylcholine, and immediately loaded onto a CaM-Agarose column, equilibrated with buffer B, supplemented with 100 mM KCl and 50 μM $CaCl_2$. The column was washed with modified buffer B (100 mM KCl, 50 μM $CaCl_2$, and 0.05% $C_{12}E_8$). Proteins bound to calmodulin were eluted with the same buffer, but

containing 0.5 mM or 5 mM EGTA instead of $CaCl_2$. The flow rate during elution was 0.2 ml/min. Proteins were monitored spectrophotometrically at 280 nm. The fractions were assayed for protein concentration, supplemented with 400 μg/ml phosphatidylcholine, and assayed for ATPase activity. Ca^{2+}-ATPase was eluted with 0.5 mM EGTA. Fractions with the highest activity were pooled and stored at −80°C, without losing of activity for at least 6 months. The concentration of protein was measured using BioRad Protein Assay kit.

2.3. Enzyme activity assay

Ca^{2+}-ATPase activity was measured by colorimetric determination of P_i hydrolyzed from ATP, by the method of Lin and Morales [20]. The activity was expressed as μmoles of P_i liberated by 1 mg of protein per hour. The reaction mixture contained: 50 mM Tris–HCl (pH 7.4), 100 mM KCl, 3 mM $MgCl_2$, 75 μM $C_{12}E_8$, 3 mM ATP, 1 mM EGTA, and 1.020 mM $CaCl_2$ (10 μM Ca^{2+}-free) in a total volume of 200 μl. 0.2–0.3 g of the enzyme were incubated for 1 min at 37°C. No Ca^{2+}-independent activity was present in the purified enzyme. The characteristics of the Ca^{2+}-ATPase purified from cortical, cerebellar and hippocampal synaptosomal membranes were described in detail elsewhere [19]. The effect of the protein kinases on Ca $^{2+}$-ATPase activity was measured in the presence of 2.2 U PKA or 0.9 mU PKC (Calbiochem, Switzerland). One unit of PKA was defined as the amount of enzyme that transferred 1.0 pmol of phosphate to histone H1 per minute at 30°C, pH 6. One unit of PKC was defined as the amount of enzyme that will transfer 1.0 nmol of phosphate to histone III-S per minute at 22°C, pH 7.4. The results are expressed as percentage in relation to the control activity of the enzyme from each set of experiments, taken as 100%. One μM PMA was used as an activator of PKC, and was prepared as a stock solution in DMSO. The final concentration of DMSO in samples did not exceed 0.2%, and this concentration had no influence on Ca^{2+}-ATPase activity.

2.4. [γ-^{32}P]ATP phosphorylation of Ca^{2+}-ATPase by PKA and PKC

The phosphorylation reaction was carried out according to the method described previously, with some modification [17]. The reaction was performed for 30 min at 30°C in the standard reaction mixture containing the purified Ca^{2+}-ATPase, as described in the figure legends, 50 mM Tris–HCl (pH 7.4), 50 μM sodium vanadate, 100 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA, and 22 U of PKA or 9 mU of PKC, in total volume of 30 μl. 1.5 mM $CaCl_2$ and 1 μM PMA were included as activators of PKC. Phosphorylation reaction was started by the addition of 90 μM [γ-^{32}P]ATP (200–300 cpm/pmol), and terminated by the addition of SDS-buffer containing 50 mM Tris–HCl (pH 6.8), 1% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 5% (w/v) sucrose and 0.05% (w/v) bromophenol blue. Polyacrylamide gel electrophoresis in the presence of SDS (SDS–PAGE) was performed on slab gels consisting of 7.5% acrylamide separation gel, and a 3% stacking gel according to the method of Laemmli [21]. The running buffer was 25 mM Tris–HCl (pH 8.3), 192 mM glycine, and 0.1% SDS. After electrophoresis, gels were stained with silver or Coomassie blue, and exposed by X-ray films (Foton) with intensifying screen. Shift in electrophoretic mobility of phosphorylated Ca^{2+}-ATPase was determined using the VHS Image Master system (Pharmacia LKB). The level of ^{32}P incorporation was measured by counting for Cerenkov radiation of bands excised from gels, and the stoichiometry was calculated taking into account the amount of ^{32}P incorporated into Ca^{2+}-ATPase, the amount of Ca^{2+}-ATPase, and specific activity of [γ-^{32}P]ATP.

2.5. Immunoassays

2.5.1. Immunodetection of Ca^{2+}-ATPase

Six different Ca^{2+}-ATPase antibodies raised against human enzyme were used in this study. General antibodies which recognized all isoforms of Ca^{2+}-ATPase were: monoclonal 5F10 (Sigma), generated against amino acid residues 724–783 of erythrocyte calcium pump, and polyclonal 2A, raised against amino acids 765–835 of human isoform 2 [2,22]. Procedures for generation and purification of four polyclonal antibodies 1N (residues 1–88), 2N (residues 1–96), 3N (residues 1–83), and 4N (residues 1–84) raised against the N-terminal portions of the human isoforms PMCA1, PMCA2, PMCA3 and

PMCA4, respectively, were described in [2]. For Western blotting, 2–3 μg of the enzyme were separated by 7.5% SDS–PAGE and transferred to Immobilon PVDF membrane in buffer containing 20 mM Tris, 192 mM glycine and 20% methanol at 30 V overnight. Non-specific binding was blocked by 5% non-fat dry milk for 2 h at room temperature, and next the membrane was incubated for 1 h with monoclonal antibody-5F10 (diluted 1:100 in TBS with 1% non-fat dry milk), or with polyclonal antibodies 2A (1:500), 1N (1:500), 2N (1:500) and 3N (1:500). For staining, the goat anti-mouse (1:3000) or mouse anti-rabbit (1:80 000) IgG-coupled alkaline phosphatase conjugate were used. The reaction was developed with NBT and BCIP, according to manufacturer's procedure.

2.5.2. Immunodetection of P-Ser and P-Thr

For dot-blotting experiments 0.2, 0.4, 0.8, 1.2, and 2 μg of purified cortical, cerebellar and hippocampal Ca^{2+}-ATPase, and Ca^{2+}-ATPase phosphorylated by PKA or PKC, were spotted onto Immobilon PVDF. The strips were blocked with 5% non-fat dry milk for 1 h, and next incubated with the monoclonal antibodies against P-Ser or P-Thr. After incubation with alkaline phosphatase-conjugated secondary antibody, the color was developed using NBT and BCIP solutions. The quantification of the phosphoamino acids was performed by densitometry scanning of the selected areas using the VHS Image Master system. Non-specific binding was measured using the same procedure, but without Ca^{2+}-ATPase and protein kinases in the samples. Specific binding was calculated by subtracting the nonspecific binding from the total binding. The results were corrected for the background values (with or without protein kinases, respectively), and are expressed in arbitrary units.

2.6. Statistics

All data presented in the text and figures are from four to six experiments performed in duplicate, and are expressed as the mean ± S.D. The statistical calculations were carried out using Student's *t*-test. *P*-Values of less than 0.05 were considered to indicate statistically significant differences.

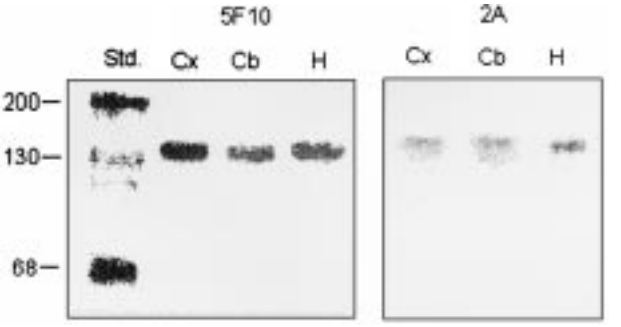

Fig. 1. Immunodetection of cortical (Cx), cerebellar (Cb) and hippocampal (H) Ca^{2+}-ATPase by general antibodies. Three μg of purified Ca^{2+}-ATPases from three rat brain regions were resolved by 7.5% SDS–PAGE, electrotransferred onto Immobilon PVDF membrane, and immunodetection was carried out with monoclonal antibody 5F10 or polyclonal antibody 2A. The molecular masses indicated at the left side of the figure represent kaleidoscope prestained standards (BioRad) transferred onto Immobilon PVDF membrane: myosin (200 kDa), β-galactosidase (130 kDa), bovine serum albumin (68 kDa).

3. Results

3.1. Immunocharacteristics of Ca^{2+}-ATPases purified from cortical, cerebellar and hippocampal synaptosomal membranes

Synaptosomal membranes from rat cortex, cerebellum and hippocampus obtained by a Ficoll-gradient centrifugation were solubilized with 0.5% $C_{12}E_8$, and the Ca^{2+}-ATPase was purified by two-step chromatography [19]. The purification yielded the enzyme in monomeric form, with a very high and stable specific activity, which was more than 150 times higher than that observed in the synaptosomal membranes. Equal amounts of purified Ca^{2+}-ATPase from cortical, cerebellar and hippocampal synaptosomal membranes were separated by 7.5% SDS–PAGE, electrotransferred on Immobilon PVDF membrane, and incubated with the two antibodies that recognize the four PMCA isoforms, i.e., the monoclonal antibody 5F10 or polyclonal antibody 2A. The epitope of 5F10 and 2A is located in a highly conserved region of the cytosolic domains of the PMCAs (724–783 amino acids and 765–835 aa, respectively). These two antibodies interacted with the enzyme from three brain areas with similar intensity (Fig. 1). We have also characterized the PMCA isoforms

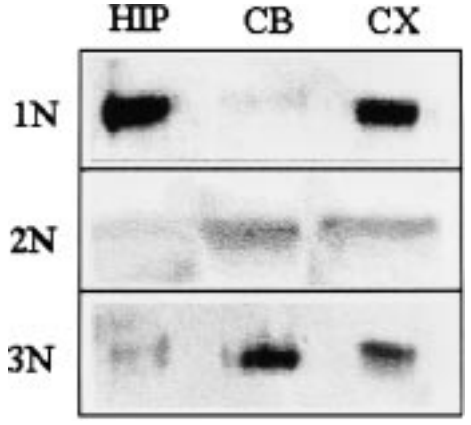

Fig. 2. Immunodetection of hippocampal (HIP), cerebellar (CB) and cortical (CX) Ca^{2+}-ATPase isoforms. Three μg of purified Ca^{2+}-ATPases from three rat brain regions were resolved by 7.5% SDS–PAGE, electrotransferred onto Immobilon PVDF membrane, and immunodetected with polyclonal antibodies raised against N-terminal parts of PMCA1 (1N), PMCA2 (2N) and PMCA3 (3N) isoforms.

presence of in purified preparations using the antibodies generated against N-terminal portions of four Ca^{2+}-ATPase isoforms [2]. Very high immunoreactivity was observed for PMCA1 with hippocampal and cortical Ca^{2+}-ATPases, whereas a lower level of immunoreactivity was detected in the case of the cerebellar enzyme preparation (Fig. 2). PMCA2 was present in nearly equal amounts in cerebellum and cortex, whereas in hippocampus the signal of 2N antibody was approximately three times lower. The amount of PMCA3 isoform decreased in the order cerebellum, cortex, hippocampus. No staining with the antibody against PMCA4 isoform was found in the examined preparations (data not shown), but this antibody was reported to react only with the human tissue [2].

3.2. *Effect of protein kinases A and C on the Ca^{2+}-ATPase activity*

The basal activity of purified enzymes was in the range 210–240 μmol P_i/mg per hour for cortex, 290–340 μmol P_i/mg per hour for cerebellum, and 350–410 μmol P_i/mg per hour for hippocampus. To compare the effect of phosphorylation on the activity of purified Ca^{2+}-ATPase from three regions of rat brain, the reactions were performed under the appropriate conditions for PKA and PKC, respectively. The action of protein kinases differed in the examined brain areas (Fig. 3). The cortical Ca^{2+}-ATPase activity was significantly enhanced after incubation with protein kinase A (290%), and protein kinase (250%). PKA and PKC stimulated the cerebellar Ca^{2+}-ATPase to a lesser degree, although the activity was still high (140%–160%). The increase of activity was also observed for hippocampal enzyme phosphorylated by PKA (130%), and PKC (170%). It

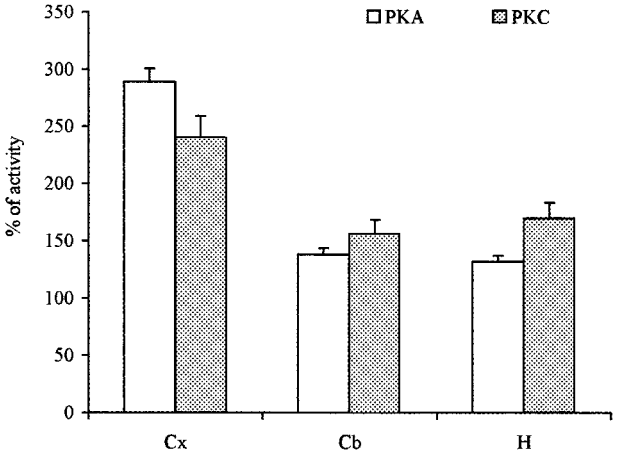

Fig. 3. The effect of protein kinases A and C on the Ca^{2+}-ATPase activity. The cortical (Cx), cerebellar (Cb) and hippocampal (H) Ca^{2+}-ATPases were incubated in standard reaction mixture with PKA or PKC. The results are expressed as percentage in relation to the control activity of enzyme, taken as 100% ($P < 0.05$).

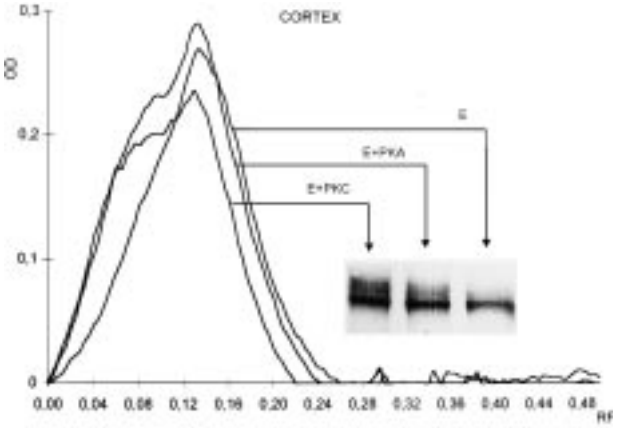

Fig. 4. Electrophoretic shift of cortical Ca^{2+}-ATPase. Calcium pump was incubated with or without of protein kinases, and separated on 7.5% SDS–PAGE. Each line contained 5 μg of purified enzyme. The migration of the native (E) and phosphorylated Ca^{2+}-ATPase (E+PKA, E+PKC) was estimated by densitometry scanning of the Coomassie blue-stained gel.

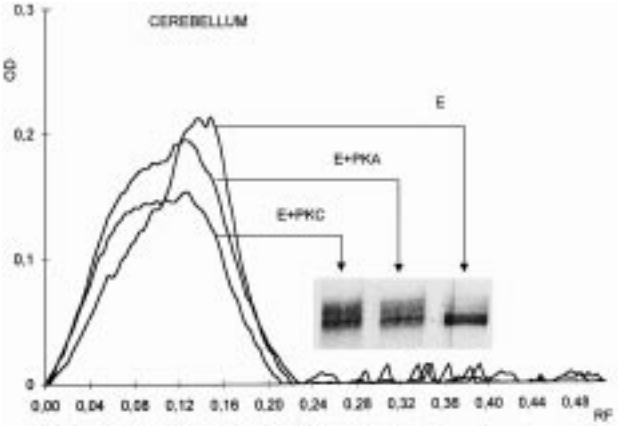

Fig. 5. Electrophoretic shift of cerebellar Ca^{2+}-ATPase. Each line contained 4 μg of purified enzyme. Other details are the same as in Fig. 4.

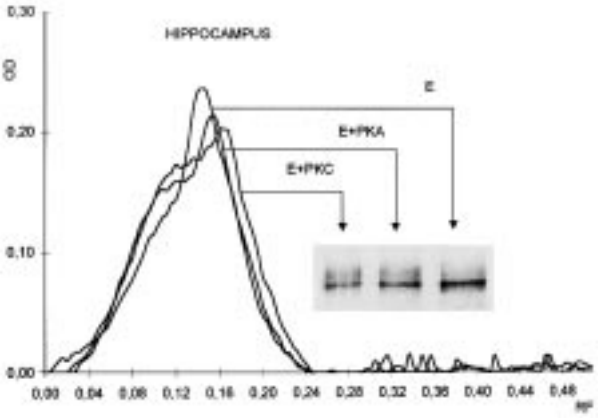

Fig. 6. Electrophoretic shift of hippocampal Ca^{2+}-ATPase. Each line contained 2.5 μg of purified enzyme. Other details are the same as in Fig. 4.

should be noted that the basal activity of cortical, cerebellar and hippocampal Ca^{2+}-ATPases differed, but reached the similar values after the phosphorylation.

3.3. Electrophoretic shift of phosphorylated Ca^{2+}-ATPase

The electrophoretic mobility of purified Ca^{2+}-ATPases from cortex, cerebellum and hippocampus incubated in the presence of PKA and PKC was altered. Representative results are shown in Fig. 4–6. In control samples, the Ca^{2+}-ATPases were incubated in parallel under the same conditions, but without protein kinases. The major peak of the phosphorylated cortical Ca^{2+}-ATPases appeared more asymmetrical than before exposure to the protein kinases, and the profile of PKC or PKA phosphorylated Ca^{2+}-ATPase differed (Fig. 4). The cerebellar, native enzyme ran as apparently multiple polypeptide components of M_r 130–138 kDa. The phosphorylation by PKA and PKC affected the mobility of Ca^{2+}-ATPase in SDS gels differently (Fig. 5). For hippocampal Ca^{2+}-ATPase, the electrophoretic pattern changed in a similar way for PKC and PKA-phosphorylated samples (Fig. 6).

3.4. The stoichiometry of ^{32}P incorporation

Typical autoradiograms of ^{32}P-labeled enzymes are presented in Fig. 7. No radioactivity occurred without PKA or PKC presence in the reaction mixture (data not shown). In standard phosphorylation conditions the highest rate of ^{32}P incorporation was observed for cortical Ca^{2+}-ATPase (0.4–0.6 pmol/pmol for PKA and 0.5–0.7 pmol/pmol for PKC). In cerebellum, PKA and PKC incorporated 0.4–0.5 pmoles

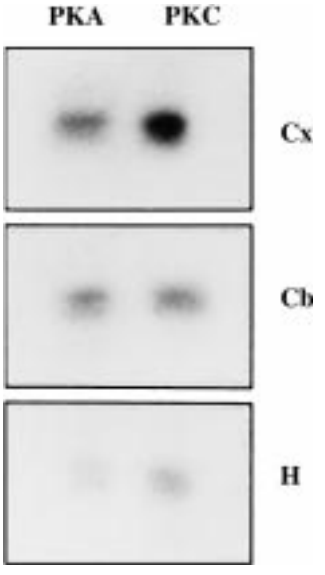

Fig. 7. Phosphorylation of cortical (Cx), cerebellar (Cb) and hippocampal (H) Ca^{2+}-ATPase by PKA and PKC. 2.5–3 μg of ^{32}P-labeled proteins after SDS–PAGE were autoradiographed with the intensifying screen. After autoradiography, the bands representing Ca^{2+}-ATPase were excised from gels, and the amount of incorporated radioactive phosphate was determined in a scintillation counter. No incorporation was observed without PKA or PKC in the reaction mixture (data not shown).

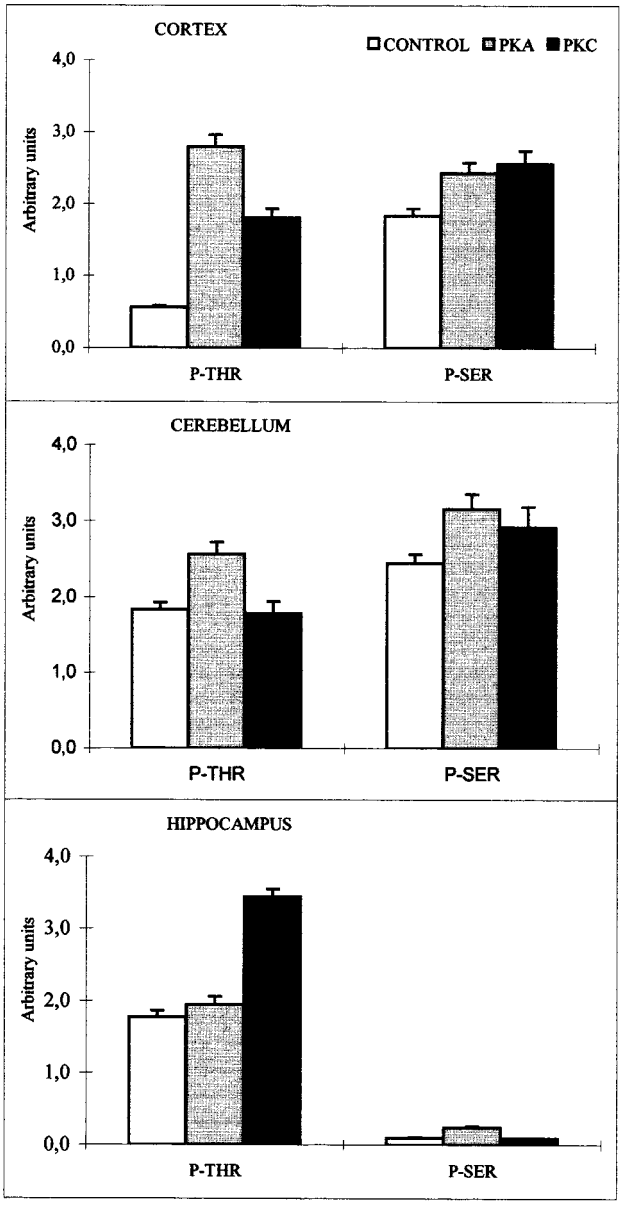

Fig. 8. Quantification of phosphoserine and phosphothreonine. Dot-blotting of the control and phosphorylated by PKA and PKC Ca^{2+}-ATPase was performed as described in Section 2. Quantification of the phosphoamino acids was done after densitometric scanning of blots using the VHS Image Master system. Bars represent a net presence of P-Ser and P-Thr in the samples.

of ^{32}P/pmol of Ca^{2+}-ATPase. The lowest level of phosphorylation was observed for hippocampal Ca^{2+}-ATPase, and the amount of ^{32}P was 0.2–0.3 pmol/pmol for PKA, and 0.3–0.4 pmol/pmol for PKC mediated phosphorylation. Since we had found that the incorporated radioactivity differed slightly from preparation to preparation, we decided to investigate whether the Ca^{2+}-ATPase has been purified in a phosphorylated state.

3.5. Immunodetection of P-Ser and P-Thr

Studies using antibodies against P-Thr and P-Ser revealed that both phosphorylated amino acids were present in the purified, native enzyme (Fig. 8). In cerebellar and hippocampal enzymes phosphothreonine was present at the similar level, whereas in cortex the signal was low. Cortical and cerebellar Ca^{2+}-ATPase exhibited the highest level of phosphoserine, whereas the staining with anti-P-Ser in the hippocampal enzyme showed no reaction. Phosphorylation by PKA significantly increased the amount of P-Thr in cortex, although we have also observed an increase of P-Ser. In cerebellum after incubation with PKA similar amounts phosphothreonine and phosphoserine residues were found. A very little, but statistically significant, PKA-mediated increase of both phosphoamino acids was detected in hippocampal enzyme. After phosphorylation of Ca^{2+}-ATPase by protein kinase C, a marked enhancement of the signal for P-Thr and P-Ser was observed in cortical enzyme. Protein kinase C preferentially phosphorylated P-Ser in cerebellar, and P-Thr in hippocampal Ca^{2+}-ATPase.

4. Discussion

The work presented here investigated the influence of phosphorylation process mediated by PKA and PKC on regulation of Ca^{2+}-ATPase activity in the three selected regions of rat brain. Ca^{2+}-ATPase purified from cortical, cerebellar and hippocampal synaptosomal membranes was phosphorylated in vitro by protein kinase A and protein kinase C. Using the isoform-specific polyclonal antibodies we have also characterized the presence of PMCA isoforms in the enzymes purified from synaptosomal membranes.

In rat brain the four PMCA isoforms are present. The complete amino acid sequences derived from cDNA have been obtained for the rat PMCA1, PMCA2, PMCA3 and PMCA4 [23–25]. In addition, several alternative spliced variants have been reported [26]. The distribution of isoforms PMCA

mRNAs studied by in situ hybridization revealed that in most regions of rat brain the intensity of the signal was in the order PMCA1 > PMCA2, 3 ≫ PMCA4 [3,27,28]. In different rat brain areas, the existence of multiple PMCA isoforms at the protein level has been recently shown with antibodies directed against N-terminal region of human PMCAs, as well as using variant-specific polyclonal antibodies that distinguished the alternatively spliced variants of the four PMCA isoforms [5,6]. We have observed similar distribution of PMCA1, PMCA2 and PMCA3 isoforms in the enzymes purified from synaptosomal membranes of rat brain. A similar pattern could suggest that the calcium pump is concentrated mainly in nervous endings, and it could have profound consequences on neuronal signaling. We have found that hippocampal Ca^{2+}-ATPase comprised mainly the PMCA1 isoform, and minor amounts of the PMCA2 and PMCA3 isoforms. In cortical nervous endings PMCA1 was also the major isoform, but higher levels of PMCA2 and PMCA3 than in hippocampus were present. The cerebellar Ca^{2+}-ATPase contained the highest amount of PMCA3 and PMCA2, but very little immunoreactivity of PMCA1. We have also observed some discrepancy in PMCA2 detection in cerebellar enzyme. PMCA2 was reported to be expressed at high level in rat brain, particularly in Purkinje cells. However, we have found less immunoreactivity than we had expected. A similar low level of PMCA2 has been observed in microsomal fraction of rat cerebellum [6]. One possible explanation is that the synaptosomal membranes besides Purkinje cells contained nervous endings from granular and molecular layers, which have different composition of PMCA isoforms. Thus, our preparation could reflect some 'average' immunoreactivity for cerebellar enzyme. We have not detected the PMCA4 isoform in the examined rat brain areas, but the antibody used has been reported to react only with human tissues. In our previous study we detected some immunoreactivity in purified cerebellar Ca^{2+}-ATPase using monoclonal JA9 antibody which recognized PMCA4 isoform [12,17]. Northern blot analysis revealed that rat brain contained a low amount of PMCA4 mRNA, but at the protein level isoform 4 was abundant in crude microsomal fraction from frontal cortex, with less present in hippocampus and cerebellum [6,25]. Taken together, the unique pattern of PMCA isoforms at the protein level, confirmed independently by several laboratories, could be critical for understanding of functional diversity of rat brain areas.

This is the first report showing that the serine/threonine protein kinase-mediated phosphorylation of synaptosomal Ca^{2+}-ATPase appears to be physiologically significant, since the purified calcium pump contained phosphoamino acids, i.e., P-Ser and P-Thr. This indicates that phosphorylation of the pump isoforms is a naturally existing process in neuronal cells, although the nature of protein kinases that phosphorylates the Ca^{2+}-ATPase in vivo is not known. Moreover, the activity of enzyme purified from cortical synaptosomal membranes was greatly enhanced after incubation with PKA and PKC. Under the same experimental conditions the cerebellar and hippocampal enzymes were activated to a lesser degree. However, it should be noted that the basal activity, as well as the phosphorylation state of purified enzymes, were different. Under the in vitro assay, PKA and PKC further activated the calcium pump from three brain areas almost to the same value. PKC belongs to the kinase family consisting of at least 11 closely related isoenzymes, which exhibit distinct tissue distribution [29]. It is also well documented that some substrates are phosphorylated by calcium-dependent isoenzymes of PKC, whereas the others are good substrates for calcium-independent ones. PKC preparations used in our experiments were a mixture of rat brain isoforms, thus the different selectivity of PKC isoenzymes towards PMCA isoforms cannot be excluded. These double specificities could be therefore crucial for the efficiency of Ca^{2+}-induced signaling events in vivo, as well as for the maintenance of calcium homeostasis in nervous cell.

The stoichiometry of phosphorylation was also significantly different in Ca^{2+}-ATPase from three examined brain regions. However, it is difficult to establish whether every phosphorylated site under the condition used is a 'regulatory' site, and could influence the activity of the enzyme. The phosphorylation of Ser and Thr was previously demonstrated in purified red blood cell Ca^{2+}-ATPase phosphorylated with rat brain type III PKC [12]. Using a synthetic peptide, corresponding to the calmodulin-binding

domain of the pump, it was shown that the threonine was phosphorylated by protein kinase C [30]. Recent studies also demonstrated that at least one serine residue located carboxy-terminally to the CaM-binding domain was phosphorylated by PKC [12]. The study performed with isoform PMCA2 and PMCA3 variants which were expressed in COS cells revealed that PKC regulated their activity in different way [31]. Little or no phosphorylation by PKC was detected in PMCA2b and PMCA3b forms. PMCA2a and -3a variants were phosphorylated, without, however, increasing the Ca^{2+} transport activity. The phosphorylation process prevented stimulation of Ca^{2+}-ATPase by calmodulin, and authors suggest that PKC would inhibit the activity of these isoforms in the cell. In our preliminary studies we have also observed the diminished binding of Texas Red-labeled calmodulin to the PKC-phosphorylated cerebellar and hippocampal Ca^{2+}-ATPases, whereas the cortical enzyme has bound the calmodulin independent of the phosphorylation state (in preparation).

We have noted some discrepancy for PKA-mediated phosphorylation of Ca^{2+}-ATPase, particularly for the hippocampal enzyme. PKA was described to phosphorylate the serine residues located to the calmodulin-binding domain of PMCA1 isoform [32]. On the other hand, using reverse transcription followed by PCR, Khan and Grover have reported the existence in the brain of potentially insensitive to PKA isoform of PMCA1 [33]. In comparison to other rat tissues, the transcripts encoding the potentially insensitive PMCA1 isoform in brain comprised about 50%. In line with these results we assume that the PMCA1-immunoreactivity detected in our preparations contains PMCA1 isoforms sensitive (cortex, cerebellum) and insensitive (hippocampus) to PKA phosphorylation.

An unexpected observation was also the increase of P-Thr-immunoreactivity after phosphorylation by PKA. Several studies have shown that among PMCA isoforms only PMCA1 possess a phosphorylation site within sequence Lys–Arg–Asn–Ser–Ser for PKA phosphorylation, and no Thr-containing site has been reported [33]. However, these results were primarily obtained with non-neuronal tissues. For example, in the erythrocyte calcium pump which is the main model for functional and structural studies, only PMCA1 and PMCA4 were present [26,34]. In addition, no PKA phosphorylation data concerning PMCA2 and PMCA3, brain-specific isoforms, are available. Thus, the PKA-dependent phosphorylation of threonine residue(s) is likely to be related to the presence of other isoforms than PMCA1. Another explanation is that the experimental conditions used could alter the conformation of the Ca^{2+}-ATPase, thereby exposing the additional sites of phosphorylation which could not be phosphorylated in vivo.

Protein kinase A-mediated phosphorylation of Ca^{2+}-ATPase could have physiological consequences on neuronal cells, since protein kinase A can be activated independently of intracellular calcium concentration. For example, Ca^{2+}-independent stimulation of adenylyl cyclase via G_S-coupled receptors could induce the accumulation of cAMP. If PKA directly catalyzes the phosphorylation of Ca^{2+}-ATPase, the activity of calcium pump will be enhanced, even if the Ca^{2+} concentration inside the cell is low. A possible hypothesis is that the calcium pump can exist in a phosphorylated state, dependent on the net intracellular phosphatases and kinases activities, which are regulated by several second-messenger operating systems. The higher level of cerebellar and hippocampal calcium pump phosphorylation in vivo could be related to specific Ca^{2+}-modulated processes named long-term potentiation (LTP) and long-term depression (LTD), which are especially connected to protein kinase and phosphatase activities [35–38]. LTP induction appears to involve the PKC action. The insertion of PKC into the membrane, and its subsequent degradation by calpain, generates a Ca^{2+}-independent kinase that has been proposed to be associated with the maintenance of LTP. Thus, in turn, PKC could phosphorylate a number of substrates (also plasma membrane calcium pump) in Ca^{2+}-dependent or Ca^{2+}-independent manner.

In conclusion, the results presented in this report reveal the differential isoenzymes composition of plasma membrane Ca^{2+}-ATPase purified from nervous endings of rat cortex, cerebellum and hippocampus, as well as various susceptibility of the enzyme for phosphorylation reactions mediated by protein kinases A and C. More importantly, these observations strongly suggest that the phosphorylation of Ca^{2+}-ATPase is the naturally existing and region-de-

pendent phenomenon in rat brain. The plasma membrane in nervous tissue contains a great number of receptors and ion channels regulating the Ca^{2+} influx. Transient increase in intracellular Ca^{2+} concentration acts as a multipotent signal for many neuronal functions [39,40]. For this reason calcium homeostasis is precisely controlled by several intracellular and membrane systems, among them the plasma membrane calcium pump. The toxic effect of increased calcium concentration inside leads to the cell death, as has been well documented in many studies [41,42]. Thus, the persistent high activity of calcium pump regulated by many ways, including phosphorylation by protein kinases, appears to be the most important feature of the system responsible for the functioning of nervous cells.

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